Farber Winners Abstracts

Similarities of Akt Activation in Cutaneous Wound Repair and Skin Carcinogenesis

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The putative CD34+/K15+ stem cells of the skin, which have been identified specifically within the ''bulge region'' of hair follicles, have recently been shown to be able to reconstitute all lineages of cells within the epidermis, hair follicles, and sebaceous glands within the skin. Although these cells are believed to play an important role during the normal regeneration of the skin and in the wound healing process, there have been no studies to define the location of stem cells during wound healing, nor have there been any reports identifying the signal-transduction pathways by which this stem cell population persists in the skin during homeostasis, wound repair, and development of skin tumors. One signaling molecule shown to be involved in both survival and proliferation of normal cells as well as tumor cells is Akt-1/protein kinase B.

The goal of the present study was to identify cell populations that contain activated Akt-1, as determined by the detection of phosphorylation of Akt at serine-473 (pAkt), over the time course of murine wound repair following full-thickness wounding. These studies used a combination of digital image analysis, immunohistochemistry, and triple-color immunofluorescence techniques to locate the stem cell populations within the skin and to quantitate the pAkt during wound healing.

In normal mouse skin, CD34+/K15+ stem cells were localized within the bulge region of hair follicles. At 24 and 48 hours, Akt-1 phosphorylation was present in epidermal keratinocytes adjacent to the wound site compared to significantly fewer cells containing pAkt at sites proximal and distal to the wound. At day 5, the majority of epidermal keratinocytes within the wound site contained pAkt, which was located in both the nuclear and cytosolic regions of each cell. The cells containing pAkt had the appearance of streaming into the wound bed. At day 9, re-epithelialization of the wound site was complete, and the majority of epidermal keratinocytes within the basal and suprabasal regions adjacent to the wound site did not contain pAkt. At this time point, levels of this signaling molecule were comparable to pAkt in keratinocytes in unwounded skin.

These observations are the first to characterize the temporal sequence of Akt-1 activation within specific skin cell populations, including the CD34+/K15+ stem cells, over the time course of wound repair and regeneration. Given the ability of Akt to regulate cell survival and proliferation, the results of the present studies suggest that Akt-1 is a critical event that occurs at early times during wound repair. The activation of Akt-1 within these specific cell populations may provide the putative ''stem cells'' that migrate into the leading edge of the wound with the ability to resist terminal differentiation and to undergo the necessary proliferation to complete re-epithelialization of the wound site. These results suggest that Akt-1 may be one pathway by which CD34+/K15+ skin stem cells survive during wound healing, thus maintaining their ability to serve as a self-renewing population of cells during wound repair of the skin. Our observation that Akt-1 is activated in CD34+/K15+ stem cell populations during wound repair is similar to our results demonstrating that Akt-1 activation occurs at early times during the development of pre-malignant skin papillomas within the CD34+/K15+ cell population. Taken together, these results lend support to the hypothesis that the Akt-1 signaling pathway may be common to both wound repair and skin carcinogenesis.

Nrf Transcription Factors are Crucial for Skin Tumor Prevention but not for Wound Healing

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The Nrf2 transcription factor is a key player in the cellular stress response through its regulation of various cytoprotective genes. In this study, we determined the role of Nrf2-mediated gene expression in keratinocytes for skin development, wound repair, and skin carcinogenesis. To overcome a possible compensatory effect exerted by the related Nrf1 and Nrf3 transcription factors, we chose to express a dominant-negative Nrf2 mutant in the epidermis of transgenic mice. The functionality of the transgene was proven *in vitro* and *in vivo*. Although primary keratinocytes from these animals revealed an enhanced rate of apoptosis, in particular in response to oxidative stress, no abnormalities of the epidermis were observed *in vivo*. Most surprisingly, the healing process of full-thickness excisional wounds proceeded normally in the transgenic animals. However, the onset, incidence, and multiplicity of chemically induced skin cancers were strikingly enhanced. This finding reveals a crucial role of Nrf-mediated gene expression in the prevention of skin tumors and suggests that activation of Nrf2 in keratinocytes could be explored to prevent carcinogenesis of this highly exposed organ.

Regulation of Vascular Endothelial Growth Factor-A Protein Activity During Wound Repair

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Vascular endothelial growth factor-A (VEGF-A) is a potent angiogenic mediator in tissue repair. Whereas VEGF-A levels are regulated through transcriptional control and mRNA stability, VEGF-A protein activity can be regulated by proteolytic mechanisms and interaction with other extracellular molecules. We hypothesize that VEGF-A protein activity is impaired in chronic non-healing wounds and is in part responsible for the reduced and disturbed angiogenic response in non-healing wounds. We analyzed VEGF-A expression, protein stability, and the presence of the most potent endogenous VEGF-A inhibitor, the soluble form of the VEGF receptor-1, in non-healing *versus* healing human wounds. VEGF-A mRNA expression was highly upregulated in non-healing wounds. Interestingly, the stability of VEGF165 protein was significantly reduced in wound fluid obtained from non-healing wounds. Protease inhibitor studies, protein sequencing, and matrix-associated laser deserption/ionization time-of-flight mass spectrometry of VEGF cleavage products, as well as a plamin-resistant VEGF165 mutant indicated that plasmin is one of the serine proteinases critically involved in this degradation process. In addition, soluble form of the VEGF receptor-1 mRNA expression and protein levels were highly upregulated in non-healing *versus* healing wounds. Only in those chronic wounds, which entered a phase of granulation tissue formation and finally wound closure, wound-healing progression correlated significantly with a decline in soluble form of the VEGF receptor-1 levels. Our studies provide two novel mechanisms as how to VEGF-A protein activity might be inhibited in non-healing *versus* healing wounds and provide the basis for novel VEGF-based therapies.

Fibroblast Growth Factor Receptor 2-IIIb is a Critical Mediator of Intercellular Signalling in Development and Disease

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Recent data have shown fibroblast growth factor receptor 2-IIIb (Fgfr2b) to be crucial for the development of the epidermis and hair follicles in the mouse fetus. As Fgfr2b-null mice die at birth, we have used a Cre-Lox approach to delete Fgfr2b specifically in keratinized epithelia. This has allowed us to determine the roles played by Fgfr2b in the adult, where previous studies have implicated it as a key player in tissue repair.

Mice lacking Fgfr2b in the skin survive into adulthood and are fertile, but display striking abnormalities in the development of their epidermis and epidermal appendages. However, when challenged by full-thickness wounding, the mice show no significant defect in their capacity for repair. Fgfr2b has been suggested to play a tumor suppressive role in the urothelium, and here we present data that show mice lacking Fgfr2b in the epidermis develop epidermal dysplasia with age, and are exquisitely sensitive to chemically induced skin carcinogenesis. Thus, although other Fgf receptors may compensate for Fgfr2b during the repair process, there is a continued requirement for Fgfr2b during postnatal development, where it acts as a key player in epithelial homeostasis.

Overexpression of Smad7 in Keratinocytes Accelerates Cutaneous Wound Healing

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Transforming growth factor β has both positive and negative effects on cutaneous wound healing. Smad7 acts as a major downstream antagonist of transforming growth factor β signaling in keratinocytes and its role in wound healing has not been defined. We have established a Smad7 transgenic mouse line using a keratin 5 (K5) promoter (K5.Smad7), which expresses Smad7 transgene at a mild level ($\sim$2-fold of the endogenous Smad7 in the skin). These mice did not have overt skin defects as shown from our previous Smad7 transgenic mice expressing much higher levels of the Smad7 transgene (*EMBO J* 2002;21:2580–2590). K5.Smad7 mice from the above low expressor line and non-transgenic littermates were subject to 6 mm full-thickness excisional wounding. K5.Smad7 mice exhibited early scab rejection, reduced inflammation, and accelerated re-epithelialization as compared with non-transgenic mice. In order to study stage-specific effects of Smad7 on wound healing, we generated a transgenic model in which Smad7 transgene expression can be induced in the epidermis and hair follicles (gene-switch-Smad7) by topical RU486 application. Smad7 induction after excisional wounding reduced inflammatory responses through the suppression of a variety of inflammatory cytokines/chemokines in gene-switch-Smad7 mice when compared to control mice. Overexpression of Smad7 exhibited accelerated re-epithelialization, which likely correlated with increased expression of metallo-proteinases and elevated extracellular signal-regulated kinases signaling in the leading epidermal edges in gene-switch-Smad7 wounds compared to control wounds. Prolonged Smad7 induction after excisional wounding reduced dermal fibrotic response and angiogenesis in the dermis, resulting in accelerated tissue repair. We conclude that the effects of Smad7 on wound healing are likely owing to blocking the inhibitory effects of transforming growth factor β on cutaneous wound healing.

Transforming Growth Factor β1 Overexpression in Mouse Keratinocytes Delays Cutaneous Wound Healing

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Transforming growth factor β1 (TGFβ1) is a multipotent secreted cytokine that plays a pivotal role in wound healing. In the present study, we used a mouse model to examine the effects of TGFβ1 on cutaneous wound healing, wherein the latent form of TGFβ1 is expressed in basal keratinocytes of the mouse epidermis, targeted by the keratin 5 promoter (K5.TGFβ1). In wild type mice with 6 mm thickness excisional cutaneous wounds, TGFβ1 protein levels were elevated immediately after wounding, and reached a peak level on day 3 post-wounding, which was 3- to 5-fold higher than that in the non-wounded skin, and sharply declined afterwards. This elevated expression pattern suggests that only transient TGFβ1 overexpression is required for normal wound healing. In contrast, K5.TGFβ1 mice with prolonged TGFβ1 transgene expression at the level equivalent to the above peak level in wild-type mice exhibited a significant delay in healing of 6 mm full-thickness excisional wounds when compared to wild-type mice. Histological analysis of wounds revealed delayed re-epithelialization, increased inflammation, and prolonged granular tissue accumulation in K5.TGFβ1 mice. Immunohistochemistry analysis revealed excessive leukocyte infiltration in transgenic wounds when compared to wild-type wounds. Accumulation of leukocytes was also accompanied by increased expression of inflammatory cytokines, chemokines, angiogenic factors, and fibrotic factors. Proliferation at the leading edge of the migrating keratinocytes was reduced in transgenic skin compared to the non-transgenic skin, evident by proliferating cell nuclear antigen staining. To assess the direct effect of TGFβ1 on keratinocyte migration, we performed an *in vitro* migration assay and found that 5.TGFβ1 keratinocytes had delayed migration when compared with wild-type cells. Our study suggests that prolonged TGFβ1 expression in keratinocytes delays cutaneous wound healing by excessive inflammation, coupled to a direct inhibitory effect on keratinocyte proliferation and migration.

The Role of Melatonin on Scarring in an Incisional Model of Dermal Wound Healing in Rats

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The role of melatonin in wound healing has not been established. Therefore, in this study, we investigated the effect of melatonin on wound healing and scar formation. In addition, we have characterized the temporal and spatial distribution of melatonin receptors during repair.

Melatonin significantly increased the quality of scarring and the rate of repair. The receptors (MT1 and MT2) were expressed in the epidermis and macrophages on day 1 in both the treated and the control wounds. Thereafter, the expression decreased post day 3. Melatonin treatment also significantly ($P<0.01$) increased arginase activity on day 1 and inducible nitric oxide synthase activity was significantly decreased on days 1 ($P<0.01$) and ($P<0.01$), followed by a significant increase in inducible nitric oxide synthase activity on day 7. Arginase generates proline, the building block for collagen synthesis. Melatonin treatment increased arginase activity and thereby collagen synthesis from day 1. It has been reported that increases in nitric oxide production prolongs the inflammatory phase of wound healing. Melatonin significantly ($P<0.01$) decreased nitric oxide synthase activity, which resulted in improved scarring. We conclude that melatonin improves scarring and that this effect is, in part, mediated by modulation of the enzymes nitric oxide synthase and arginase.

β2-Adrenergic Receptor Antagonists are Promotogenic in Keratinocytes: Potential Therapy for Promoting Wound Re-epithelialization

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Keratinocytes play an important role in wound repair by migrating directionally from the wound edge to re-epithelialize cutaneous wounds. They solely express the β2 sub-type of adrenergic receptor (β2-AR), as do dermal fibroblasts and melanocytes, and also have the capacity to synthesize catecholamines (β2-AR agonists), generating an autocrine and paracrine mediator network in the skin.

Previously, we have reported that β2-AR activation delays the wound repair process by decreasing ERK phosphorylation and retarding keratinocyte polarization, migration, and proliferation by protein phosphatase 2A-dependent mechanisms. Striking alterations in cytoskeletal conformation and morphology were also observed. Finally, we convincingly demonstrated that β2-AR activation decreased the re-epithelialization of human and murine skin wounds.

Our work suggests that the β2-AR mediator network in the skin puts the ''brakes'' on re-epithelialization delaying barrier recovery. β2-AR antagonists block the β2-AR, preventing endogenously synthesized catecholamine-mediated β2-AR activation. Here, we demonstrate that β2-AR antagonists increase extracellular signal-regulated kinase phosphorylation, single-cell migration, and scratch wound healing. Our preliminary work demonstrates that β2-AR antagonists could have potential as therapeutic agents to accelerate the wound repair process.

Non-endothelial Effects of Vascular Endothelial Growth Factor: Implications for Wound Repair

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Vascular endothelial growth factor-A (VEGF-A) is best known for stimulating endothelial cells and promoting angiogenesis, but recent studies indicate that VEGF can also signal through its receptors in some non-endothelial cell types. Recently, we have discovered that keratinocytes express vascular endothelial growth factor (VEGFR)-1 and dermal fibroblasts express VEGFR-1 and VEGFR-2. In addition, both cell types proliferate in response to VEGF *in vitro*, suggesting VEGF may directly affect both re-epithelialization and restoration of dermal architecture during wound healing *in vivo*. To determine the importance of VEGF–VEGFR-1 interactions to keratinocytes, anti-VEGFR-1-blocking antibodies were applied topically to excisional wounds, and the effect on wound closure was assessed. VEGFR-1 antibody treatment resulted in a significant delay in re-epithelialization, which correlated with a reduction in proliferating cell nuclear antigen-positive keratinocytes at the wound edge. To determine if VEGF might influence fibroblast responses, the effect of systemic administration of neutralizing anti-VEGF antibodies on scar tissue formation was examined in incisional wounds. Neutralization of VEGF reduced the size of the scars by approximately 75% compared to control scars, suggesting that VEGF can modulate collagen deposition and/or remodeling. Together, these data imply that VEGF does not simply promote wound angiogenesis, but may also act on non-endothelial cell types to influence wound re-epithelialization and scar formation.

(Originally chosen as awardee, but was unable to attend Symposium)

P63 Isoform Switching is Required for Induction of IκB Kinase-α and Epidermal Morphogenesis

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During normal epidermal development, a shift in the balance of *p*63 isoform expression from TA- to ΔN*p*63 isoforms occurs. To determine the biological significance of this switch, we generated an epidermal-specific inducible ΔN*p*63 knockdown mouse model. Interestingly, we found that ΔN*p*63 knockdown epidermis failed to undergo terminal differentiation resulting in epidermal fragility, demonstrating the critical importance for ΔN*p*63 isoforms in epidermal terminal differentiation. In addition, we found that when commitment to terminal differentiation occurs, ΔN*p*63α directly regulates IκB kinase α (IKKα), a key regulator of epidermal, skeletal, and craniofacial morphogenesis. Furthermore, using BrdU incorporation assays, we found that in IKKα$^{-/-}$ embryos, cells within the proliferative intermediate layer failed to withdraw from the cell cycle and mature into spinous cells, demonstrating that IKKα is required for this maturation process. These data are consistent with our finding that IKKα expression levels peak at E15.5, when this transition takes place. Taken together, our data demonstrate that *p*63 isoform switching is required for the induction of IKKα and epidermal morphogenesis. Furthermore, our finding that downregulating ΔN*p*63 expression *in vivo* causes skin erosions similar to those found in a subset of ectodermal dysplasia patients provide novel insights into the molecular basis of ectodermal dysplasias.